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Evaluation of the AID ESBL line probe assay for rapid detection of extended-spectrum β -lactamase (ESBL) and KPC carbapenemase genes in Enterobacteriaceae

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Abstract: **OBJECTIVES:** This study aimed at evaluating the AID ESBL line probe assay for the detection of extended-spectrum β -lactamase (ESBL) and KPC carbapenemase genes in Enterobacteriaceae. **METHODS:** The AID ESBL line probe assay was verified for accuracy of its probes using PCR products from clinical ESBL Enterobacteriaceae strains harbouring TEM, SHV and CTX-M ESBL genes and KPC genes and mutant fusion PCR products generated from Enterobacteriaceae strains containing wild-type (wt) TEM and wt SHV. Sensitivity and specificity was determined testing a set of 424 clinical Enterobacteriaceae strains (including 170 strains negative for TEM, SHV, CTX-M and KPC to evaluate the possibility of false positive signals). **RESULTS:** The line probe assay was shown to detect with 100% accuracy ESBL genes for which oligonucleotide probes are present in the assay. Testing a set of 424 clinical Enterobacteriaceae strains showed 100% sensitivity and specificity for the detection and differentiation of TEM, SHV and CTX-M ESBL genes present in that group. In addition, the line probe assay detected KPC genes accurately. **CONCLUSIONS:** The AID ESBL line probe assay is an accurate and easy-to-use test for the detection of ESBL and KPC genes, which can readily be implemented in the diagnostic laboratory.

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Evaluation of the AID ESBL line probe assay for rapid detection of extended-spectrum β -lactamase (ESBL) and KPC carbapenemase genes in Enterobacteriaceae

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Results: The line probe assay was shown to detect with 100% accuracy ESBL genes for which oligonucleotide probes are present in the assay. Testing a set of 424 clinical Enterobacteriaceae strains showed 100% sensitivity and specificity for the detection and differentiation of TEM, SHV and CTX-M ESBL genes present in that group. In addition, the line probe assay detected KPC genes accurately.

Conclusions: The AID ESBL line probe assay is an accurate and easy-to-use test for the detection of ESBL and KPC genes, which can readily be implemented in the diagnostic laboratory.

Keywords: TEM, SHV, CTX-M, KPC, antibiotic resistance

Introduction

Increasing prevalence of resistance against cephalosporins in extended-spectrum β -lactamase (ESBL)-producing Enterobacteriaceae has continuously been reported over the past years. Depending on the ESBL type, these β -lactamases have the ability to hydrolyse various penicillins, first-, second- and third-generation cephalosporins, and aztreonam (but not cephamycins or carbapenems). Currently, the most prevalent ESBLs include TEM, SHV and CTX-M enzyme types.^{1,2} While TEM and SHV β -lactamase wild-type (wt) genes have to evolve to ESBL by mutation, all CTX-M-type enzymes naturally show ESBL activity. To date, >170 SHV, >200 TEM and ~130 CTX-M variants (clustered in five groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25) have been identified (for an update see <http://www.lahey.org/studies>). CTX-M-type β -lactamase-producing *Escherichia coli* have emerged in recent years and CTX-M enzymes are now the most prevalent ESBL type in Europe and North America.^{3,4} While *Klebsiella pneumoniae* and *E. coli* strains represent the most frequently isolated ESBL-producing species worldwide, ESBLs have been identified in several other members of the Enterobacteriaceae family, such as *Enterobacter cloacae* and *Klebsiella oxytoca*.⁵

Phenotypic susceptibility testing for ESBLs may be complicated by the presence of multiple β -lactamases in a single bacterial strain, e.g. ESBLs, AmpCs and carbapenemases, and by different levels of β -lactamase gene expression.^{6,7} Genotypic tests have the potential to accurately identify different β -lactamase genes and mutations that result in ESBLs.⁸

Based on epidemiological analyses, an ESBL line probe assay has been designed (AID Autoimmun Diagnostika GmbH, Germany) to detect the most prevalent ESBL genes present in Enterobacteriaceae in Europe and North America and the KPC carbapenemase gene (Figure 1 and Table S1, available as Supplementary data at JAC Online). The aim of this study was to evaluate the specificity and sensitivity of the AID ESBL line probe assay (AID Autoimmun Diagnostika GmbH, Germany) in clinical Enterobacteriaceae strains.

Methods

Strains, culture conditions and fusion PCR amplicons

In this study, we used a carefully characterized collection of non-duplicate, non-outbreak, clinical Enterobacteriaceae strains ($n=424$; Table S2, available as Supplementary data at JAC Online). These strains were isolated

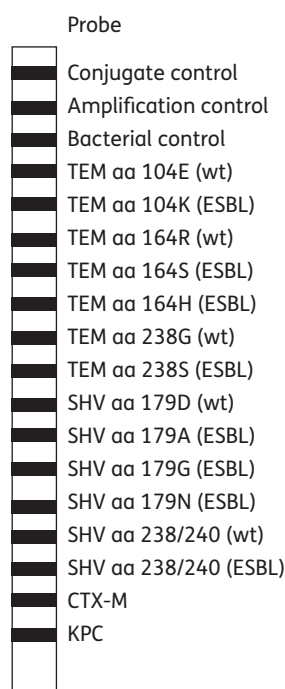


Figure 1. Schematic representation of the ESBL line probe assay (AID, Strassberg, Germany) for the detection of ESBL (TEM, SHV and CTX-M) and KPC genes in Enterobacteriaceae. TEM and SHV variants detected by the TEM and SHV mutant probes are listed in Table S1, available as Supplementary data at JAC Online.

from clinical specimens from patients from tertiary and secondary care hospitals in the Zurich metropolitan area (Switzerland, Europe) over a period of 7 months from October 2009 until April 2010. Previously, we have characterized this set of clinical strains phenotypically and genotypically for the presence of ESBL genes.⁸ Fusion PCR amplicons were generated for ESBL TEM and ESBL SHV mutations that were not available to us from clinical isolates. For details, see the Supplementary methods and Table S3 (both available as Supplementary data at JAC Online).

AID ESBL line probe assay

The AID ESBL line probe assay can detect ESBL TEM (E104K, R164S, R164H and G238S), ESBL SHV [D179A, D179G, D179N and mutant amino acids (aas) 238/240] and CTX-M genes (all known classes) (Figure 1 and Table S1, available as Supplementary data at JAC Online). In addition, a probe for one of the most prevalent carbapenemases in Europe and North America, i.e. KPC, is included.

AID ESBL line probe assay testing was performed following the manufacturer's instructions (AID Autoimmun Diagnostika GmbH, Germany). Briefly, the procedure for bacterial cultures consists of DNA extraction (laboratories are free to choose their own method), PCR amplification with primers for the ESBL and KPC target genes (~2.5 h), reverse hybridization of the PCR amplicons with the probes present on the nitrocellulose strip (~1 h) and, finally, signal development (~1 h). A DNA isolation kit and thermostable DNA polymerase are not provided in the AID ESBL kit. The kit contains primers for PCR amplification and reagents for line probe reverse hybridization and signal detection. The DNA used for PCR amplification with the biotinylated primers of the AID ESBL kit was either extracted from bacterial colonies grown on sheep blood agar medium using the InstaGene Matrix (Bio-Rad, Reinach, Switzerland; DNA extraction time ~3 h), or alternatively ~200 ng of mutant fusion PCR amplicons was used. The PCR

was performed according to the protocol provided with the line probe assay. Five units of FastStart Taq Polymerase (Roche Diagnostics, Rotkreuz, Switzerland) together with its corresponding buffer were used per PCR.

Results

Verification analysis of the AID ESBL line probe assay

The ESBL line probe assay (Figure 1) was evaluated using PCR amplicons of (i) selected, phenotypically and genotypically well-characterized clinical ESBL Enterobacteriaceae strains and (ii) mutant fusion PCR amplicons for those TEM and SHV mutations for which corresponding clinical strains were not available to us, in order to verify the accuracy of all the probes present in the assay (Table 1). The clinical strains consisted of *E. coli* wt TEM, *Proteus mirabilis* ESBL TEM 104K/238S, *K. pneumoniae* wt SHV, *K. pneumoniae* ESBL SHV 238S/240wt, *E. coli* ESBL SHV 238S/240K, *E. coli* wt TEM and CTX-M group 1, *E. coli* wt TEM, and CTX-M group 8, *Klebsiella* sp. wt SHV and CTX-M group 9 and *K. pneumoniae* wt TEM, wt SHV and KPC (Table 1). Fusion PCR amplicons from wt TEM and wt SHV genes contained introduced ESBL mutations corresponding to TEM 164S and TEM 164H and SHV 179A, 179G and 179N, respectively. The line probe assay detected PCR amplicons representing ESBL and KPC genes with 100% accuracy, with no false positive or false negative results (Table 1). The SHV 238S PCR amplicon hybridized with two probes, i.e. wt SHV 238/240 and ESBL SHV 238/240. Other cross-reactions were not observed.

Sensitivity and specificity of the AID ESBL assay in clinical strains

The sensitivity and specificity of the AID ESBL line probe assay was studied in clinical strains, using a collection of well-characterized (molecular and phenotypic) clinical Enterobacteriaceae strains ($n=424$; 227 *E. coli*, 55 *K. pneumoniae*, 19 *K. oxytoca*, 61 *E. cloacae* and 62 strains of other species; Table S2, available as Supplementary data at JAC Online). The nine clinical strains used in the verification analysis (Table 1) were part of this group. The collection contained: (i) 148 TEM-positive strains with 145 wt TEM, 2 ESBL TEM 104K/238S and 1 strain containing two TEM genes (ESBL TEM 104K/238S and wt TEM); (ii) 86 SHV-positive strains with 60 wt SHV, 6 ESBL SHV 238S and 20 ESBL SHV 238S/240K; (iii) 134 CTX-M-positive strains with 91 CTX-M group 1, 42 CTX-M group 9 and 1 CTX-M group 8; and (iv) 3 *K. pneumoniae* strains with a KPC gene.

Results show an excellent performance of the ESBL line probe assay, with a specificity and sensitivity of 100% for ESBL TEM, ESBL SHV and CTX-M detection in this group of strains (Table 2). As a negative control group, to check for the possibility of false positive signals in the line probe assay, we selected 170 clinical strains characterized phenotypically as non-ESBL and confirmed genetically as TEM, SHV and CTX-M negative. None of these strains, including three *K. oxytoca* strains with a K1 β -lactamase gene, which is related to CTX-M and overproduction of which can result in false positive ESBL phenotypic testing,⁹ produced false positive signals. In the absence of false positive or false negative ESBL results, the positive predictive value and the negative predictive value of the assay both add up to 100%.

The one *E. coli* strain with a wt TEM and an ESBL TEM gene showed hybridization with both the wt and the corresponding

Table 1. Verification analysis of the AID ESBL line probe assay using clinical strains and fusion PCR amplicons

Species/fusion PCR amplicon	TEM								SHV							CTX-M		KPC	
	AID								AID										
	wt 104E	ESBL 104K	wt 164R	ESBL 164S	ESBL 164H	wt 238G	ESBL 238S	Seq. ^a	wt 179D	ESBL 179A	ESBL 179G	ESBL 179N	wt 238/240	ESBL 238/240	Seq. ^a	AID	Seq. ^a	AID	Seq. ^a
<i>E. coli</i>	+	–	+	–	–	+	–	wt TEM	–	–	–	–	–	–	–	–	–	–	–
<i>P. mirabilis</i>	–	+	+	–	–	–	+	TEM 104K, 238S	–	–	–	–	–	–	–	–	–	–	–
TEM 164S PCR	+	–	–	+	–	+	–	TEM 164S	–	–	–	–	–	–	–	–	–	–	–
TEM 164H PCR	+	–	–	–	+	+	–	TEM 164H	–	–	–	–	–	–	–	–	–	–	–
<i>K. pneumoniae</i>	–	–	–	–	–	–	–	–	+	–	–	–	+	–	wt SHV	–	–	–	–
SHV 179A PCR	–	–	–	–	–	–	–	–	–	+	–	–	+	–	SHV 179A	–	–	–	–
SHV 179G PCR	–	–	–	–	–	–	–	–	–	–	+	–	+	–	SHV 179G	–	–	–	–
SHV 179N PCR	–	–	–	–	–	–	–	–	–	–	–	+	+	–	SHV 179N	–	–	–	–
<i>K. pneumoniae</i>	–	–	–	–	–	–	–	–	+	–	–	–	+	+	SHV 238S/240wt	–	–	–	–
<i>E. coli</i>	–	–	–	–	–	–	–	–	+	–	–	–	–	+	SHV 238S/240K	–	–	–	–
<i>E. coli</i>	+	–	+	–	–	+	–	wt TEM	–	–	–	–	–	–	–	+	CTX-M group 1	–	–
<i>E. coli</i>	+	v	+	–	–	+	–	wt TEM	–	–	–	–	–	–	–	+	CTX-M group 8	–	–
<i>Klebsiella</i> sp.	–	–	v	–	–	–	–	–	+	–	–	–	+	–	wt SHV	+	CTX-M group 9	–	–
<i>K. pneumoniae</i>	+	–	+	–	–	+	–	wt TEM	+	–	–	–	+	–	wt SHV	–	–	+	KPC

^aSeq., characterization by DNA sequencing.

Table 2. Testing of 424 clinical Enterobacteriaceae strains using the ESBL line probe assay

Strains		TEM								SHV								CTX-M		KPC	
		AID								AID											
		group	n	wt 104E	ESBL 104K	wt 164R	ESBL 164S	ESBL 164H	wt 238G	ESBL 238S	Seq. ^a	wt 179D	ESBL 179A	ESBL 179G	ESBL 179N	wt 238/240	ESBL 238/240	Seq. ^a	AID	Seq. ^a	AID
1	12	+	−	+	−	−	+	−	wt TEM	+	−	−	−	+	−	wt SHV	+	CTX-M group 1	−	−	
2	1	+	−	+	−	−	+	−	wt TEM	+	−	−	−	+	−	wt SHV	−		−	+	KPC
3	9	+	−	+	−	−	+	−	wt TEM	+	−	−	−	+	−	wt SHV	−		−	−	−
4	1	+	−	+	−	−	+	−	wt TEM	+	−	−	−	+	+	SHV 238S/240wt	+	CTX-M group 1	−	−	
5	2	+	−	+	−	−	+	−	wt TEM	+	−	−	−	+	+	SHV 238S/240wt	−		−	−	−
6	2	+	−	+	−	−	+	−	wt TEM	+	−	−	−	+	+	SHV 238S/240wt	−		−	+	KPC
7	2	+	−	+	−	−	+	−	wt TEM	+	−	−	−	−	+	SHV 238S/240K	−	−	−	−	
8	46	+	−	+	−	−	+	−	wt TEM	−	−	−	−	−	−	−	+	CTX-M group 1	−	−	
9	1	+	−	+	−	−	+	−	wt TEM	−	−	−	−	−	−	−	+		CTX-M group 8	−	−
10	11	+	−	+	−	−	+	−	wt TEM	−	−	−	−	−	−	−	+		CTX-M group 9	−	−
11	58	+	−	+	−	−	+	−	wt TEM	−	−	−	−	−	−	−	−	−	−	−	
12	1	+	+	+	−	−	+	+	wt TEM and TEM 104K, 238S	−	−	−	−	−	−	−	−	−	−	−	
13	2	−	+	+	−	−	−	+	TEM 104K, 238S	−	−	−	−	−	−	−	−	−	−	−	
14	2	−	−	−	−	−	−	−	−	+	−	−	−	+	−	wt SHV	+	CTX-M group 1	−	−	
15	1	−	−	−	−	−	−	−	−	+	−	−	−	+	−	wt SHV	+		CTX-M group 9	−	−
16	35	−	−	−	−	−	−	−	−	+	−	−	−	+	−	wt SHV	−		−	−	−
17	1	−	−	−	−	−	−	−	−	+	−	−	−	+	+	SHV 238S/240wt	−	−	−	−	
18	11	−	−	−	−	−	−	−	−	+	−	−	−	−	+	SHV 238S/240K	+	CTX-M group 9	−	−	
19	7	−	−	−	−	−	−	−	−	+	−	−	−	−	+	SHV 238S/240K	−		−	−	−
20	30	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+		CTX-M group 1	−	−
21	19	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	CTX-M group 9	−	−	
22	170	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	

The Enterobacteriaceae strains (for species see Table S2, available as Supplementary data at JAC Online) were isolated from urine, wound, respiratory tract, groin, blood culture, vagina, miscellaneous and other unspecified body sites. TEM and SHV genes were sequenced for mutation identification. Classification of CTX-M genes was based on multiplex PCRs, which differentiate between the different CTX-Ms.^{14,15}

^aSeq., characterization by DNA sequencing.

mutant probe in the line probe assay. This result was confirmed by the sequencing of TEM PCR amplicons, which demonstrated double peaks at the location of the corresponding nucleotide positions. The six strains with an ESBL SHV 238S mutation (four *K. pneumoniae* and two *E. coli* strains, see Table S2, available as Supplementary data at JAC Online) showed an equally strong positive signal for the wt SHV 238/240 probe in addition to the ESBL SHV 238/240 probe. However, for none of these strains was a double peak observed in the sequence electropherogram of the SHV PCR amplicon at the corresponding nucleic acid positions resulting in aa 238. Only the mutant nucleotide associated with ESBL SHV 238S was found, which indicates that only the ESBL SHV variant is present in these strains.

Discussion

The rapid detection and accurate identification of ESBLs is crucial for proper antibiotic therapy, to monitor resistance epidemiology and to promptly initiate hospital hygiene procedures.^{6,10,11} Although ESBL strains can be detected by phenotypic screening, the integration of genotypic methods facilitates cost-efficient and accurate ESBL detection.⁸

Genotypic screening for ESBL detection is complicated by the existence of different ESBL enzyme classes, of which TEM, SHV and CTX-M types are the most frequently occurring.^{2,5,12} Mutations resulting in ESBLs are most frequently observed in TEM aas 104, 164 and 238 and SHV aas 179, 238 and 240.¹ In general, mutation detection in TEM and SHV genes is usually done by sequence analysis¹³ and CTX-M genes can be detected by multiplex PCR.^{14,15} Few commercial systems are available for the molecular detection of ESBL genes. Microarrays developed by Check-Points (Wageningen, The Netherlands) are able to detect simultaneously TEM, SHV and CTX-M genes with a high accuracy.^{16–18} Although the assay is relatively easy to perform and many probes can be included on one array, it requires a dedicated microarray reader and software for detection and interpretation of the results. In addition, the costs per test are relatively high.¹⁸

In this study, we evaluated the performance of a new commercially available ESBL line probe assay developed by AID Diagnostika (Germany), which contains probes for the most common ESBL genes, i.e. ESBL TEM, ESBL SHV and CTX-M classes (Figure 1 and Table S1, available as Supplementary data at JAC Online). In addition, it contains a probe for the detection of KPC, which is one of the most prevalent carbapenemases in Europe.

The advantages of line probe assays are that: (i) they are easy to perform; (ii) they are already commonly used in the molecular diagnostic laboratory^{19,20}; and (iii) apart from a 'simple' PCR machine for the performance of endpoint PCRs, they do not require additional dedicated equipment. A disadvantage of line probe assays is that the amount of probes is restricted to ~15–20 probes, whereas an array can contain more probes. Costs per test of the AID ESBL array in Switzerland are less than half the price of a comparable Check-Points array.

Our results show that the line probe assay accurately detected all mutations for which probes were present (Table 1). No false negative or false positive result was observed for any of the ESBL probes. However, we consistently observed that the ESBL SHV 238S showed hybridization with both the wt and the ESBL SHV 238S probes, indicating cross-reactivity of the wt SHV 238 probe

with the ESBL SHV 238S mutant. In contrast, the ESBL SHV 238S/240K showed a hybridization signal only with the ESBL SHV 238/240 probe, but not with the wt SHV 238 probe. Cross-reactivity of the wt SHV 238 probe with the 238S mutant, but not with the 238S/240K mutant, is most likely due to the close clustering of codons 238 and 240, which hampers the design of a specific probe. However, the cross-reactivity of the wt SHV 238 probe for the ESBL SHV 238S mutant did not pose a problem in test interpretation resulting in a false negative result, as the presence of a hybridization signal for the ESBL SHV 238/240 mutant is always interpreted as positive for an ESBL SHV 238/240.

In the second part of this study, we analysed the specificity and sensitivity of the ESBL line probe assay by testing a collection of 424 clinical strains.⁸ All mutations present in the set of 424 clinical Enterobacteriaceae strains were correctly identified, resulting in 100% sensitivity of the line probe assay (Table 2). One clinical strain showed the presence of both a wt TEM and an ESBL TEM 104K/238S, which was confirmed by the presence of double peaks at the corresponding nucleic acid positions in the sequencing electropherogram. These results show that the line probe assay is able to detect at least two different TEM genes present in a single strain. Cross-reactions other than the SHV 238S with the wt SHV 238/240 probe were not observed. As with every line probe assay, the AID ESBL line probe assay is limited to the detection of ESBLs for which probes are present in the assay. In case of a phenotypic indication for the presence of an ESBL, but the detection of only wt TEM or wt SHV in the line probe assay, TEM and SHV genes can be PCR amplified followed by sequencing to resolve discrepancies. In such cases, sequence analysis will detect the presence of rare or new mutations that are not included in the line probe assay, but that result in an ESBL phenotype.

In conclusion, the ESBL line probe assay (AID) is a rapid tool for the accurate detection of the most commonly occurring ESBL genes in Enterobacteriaceae. It can either be used as a screening tool or for confirmation in case of inconclusive phenotypic test results. This assay can readily be implemented in any diagnostic laboratory possessing a 'simple' PCR machine for the performance of endpoint PCRs and is easy to use.

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Transparency declarations

E. C. B. is a consultant of AID Diagnostika. All other authors: none to declare.

Supplementary data

Supplementary methods and Tables S1 to S3 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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